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Regulation of Folate and One-carbon Metabolism in Mammalian Cells

III. ROLE OF MITOCHONDRIAL FOLYLPOLY-γ-GLUTAMATE SYNTHETASE*

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Wild-type Chinese hamster ovary (CHO) cells and CHO cell transfectants expressing human folylpoly-γ-glutamate synthetase (FPGS) activity contain mitochondrial FPGS activity of higher specific activity than the cytosolic isozyme. Expression of mitochondrial FPGS activity is required for folate accumulation by mitochondria. The mitochondrial folate pool in CHO c lls is not in equilibrium with the cytosolic pool and c ntains folylpolyglutamates of longer glutamate chain l ngth than cytosolic folates. The inability of AUX-c *li*, a CHO cell expressing high levels of *Escherichia coli* FPGS activity and containing pteroyltriglutamate, to support glycine synthesis is due to a lack of mitochondrial FPGS activity. AUX-*coli* cells lack mitoch ndrial folate despite containing high levels of cyt s lic folate.

Chinese hamster (CHO)[1] cells expressing *Escherichia coli* folylpoly-γ-glutamate synthetase (FPGS) activity and containing pteroyltriglutamates were unable to grow in the absence of glycine unless very high levels of folate were provided (1, 2). This observation was unexpected as pteroyltriglutamates effectively supported purine and thymidylate synthesis in these cells (AUX-*coli*), and serine to glycine conversion, via the serine hydroxymethyltransferase reaction, is the major provider of one-carbon moieties for folate-dependent nucleotide biosynthesis (3, 4). Mammalian cells contain cytosolic and mitochondrial serine hydroxymethyltransferase isozymes and most of the enzyme in the CHO cell is mitochondrial (4–6). The specific activity of the mitochondrial serine hydroxymethyltransferase is about 20-fold higher than the cytosolic enzyme. GlyA, a CHO mutant that lacks mitochondrial serine hydroxymethyltransferase activity, is auxotrophic for glycine suggesting that the mitochondrion is the major site of glycine synthesis in the cell (5, 7).

It has been known for some time that mitochondria contain pteroylpolyglutamates (6, 8–12) and folate-dependent enzymes (3–6, 8, 13–16) but the mechanism of folate transport and metabolism in mitochondria is not understood. FPGS is believed to be primarily, if not totally, a cytosolic enzyme (3, 17, 18). However, the phenotype of AUX-*coli* could be explained if the cell was defective in mitochondrial folate metabolism. Expression of an *E. coli* gene in mammalian cells would be expected to limit localization of the protein to the cytosol.

In this report, the subcellular distribution of folate and FPGS activity in CHO transfectants and the role of mitochondrial FPGS are described.

EXPERIMENTAL PROCEDURES

Materials—Cytochrome c, sodium hydrosulfite, *p*-iodonitrotetrazolium violet, 4-methylumbelliferyl-β-D-glucuronide, and potassium 2-oxoglutarate were from Sigma. Percoll was obtained from Pharmacia LKB Biotechnology Inc., and aliquots (40 ml) were stored in sterile polyethylene tubes (50 ml) at 4 °C. Each stock tube was handled using sterile techniques and, once opened, was used within 1 week or discarded. Other materials were obtained from standard sources or are as described in our companion studies (1, 2).

Cells and Culture Medium—The mammalian cells used and culture conditions were as described in companion studies (1, 2).

Preparation of Cell Homogenates and Subcellular Fractions—Cells were cultured in 5–10 150 cm^2 T-flasks to yield 1–2 × 10^8cells. The monolayer was rinsed with phosphate-buffered saline (4 × 5 ml, 4 °C), and then incubated with phosphate-buffered saline, 10 mM EDTA (10 ml) at room temperature until the cells detached (5–10 min). The flasks were tapped gently to dislodge the cells, and the cells were transferred to a 50-ml plastic conical tube. Cells were pelleted by centrifugation at 300 × *g* for 5 min at room temperature, and the cell pellet was washed with homogenization solution (HMS, 250 mM sucrose, 1 mM EDTA, pH 6.9, 15 ml, 4 °C). The cell pellet was resuspended in HMS (2 ml, 4 °C), and aprotinin (700 kallikrein-inactivating units) was added if enzyme assays were to be carried out. The cell suspension was transferred to a nitrogen cavitation device (Kontes) and exposed to a pressure of 36 p.s.i. for 30 min at 4 °C (19–21). The suspension of disrupted cells was collected into a 3-ml conical ground glass Duall tissue grinder and further disrupted with four strokes of the homogenizer.

Nuclei and unbroken cells were sedimented by centrifugation (900 × *g*, 6 min). The supernatant was removed carefully and transferred to another centrifuge tube and stored on ice. The pellet was resuspended in HMS (1 ml) and further dispersed by four strokes in the grinder. After centrifugation (900 × *g*, 6 min), the supernatant was combined with the first supernatant and stored on ice. The pellet was washed (3 × 1 ml HMS) and the final viscous pellet (nuclear fraction) was resuspended in HMS (1 ml). The combined supernatants were centrifuged (900 × *g*, 5 min), and any pellet was discarded. The volume of the supernatant (total post-nuclear supernatant (PNS) fraction) was increased to 5 ml by the addition of HMS.

The PNS was centrifuged (10,000 × *g*, 15 min), and the pellet was stored on ice. The supernatant was recentrifuged (10,000 × *g*, 15 min) to give a final supernatant (cytosolic fraction). The second pellet was combined with the first and was washed with HMS (2 ml) and resuspended in HMS (1 ml) to give the mitochondrial fraction.

Subcellular Fractionation on Percoll Density Gradients—A 90% stock isotonic solution of Percoll was prepared by dilution of 9 parts

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[1] The abbreviations used are: CHO, Chinese hamster ovary; FBS, fetal bovine serum; dFBS, dialyzed fetal bovine serum; FPGS, folylpoly-γ-glutamate synthetase; PNS, postnuclear supernatant; MEM, minimal Eagle's medium; HMS, homogenization solution.

of Percoll with 1 part 2.5 M sucrose containing 10 mM EDTA, pH 6.9 (19–21). The stock isotonic solution was further diluted with HMS until the refractive index was 1.3497. The starting density of the Percoll was calculated to be 1.072 g/ml. The final percent of stock isotonic solution in this solution was about 27.4% and varied slightly depending on the lot of Percoll used. A 4.0-ml cushion of 60% sucrose containing 0.002% sodium azide was overlaid with 30 ml of the diluted Percoll solution in a 40-ml polyallomer Quickseal tube. After carefully layering 6 ml of the PNS onto the Percoll, a self-forming gradient was established by centrifugation in a VTi50 rotor (Beckman) at 28,000 × *g* for 60 min (excluding acceleration and deceleration time) at 4 °C in an ultracentrifuge (18, 500 rpm, Beckman L8-M) with acceleration and deceleration settings of 9. Gradient fractions (1 ml) were collected from the bottom of the tube.

In some experiments, the PNS was fractionated on a discontinuous hybrid Percoll-metrizamide density gradient (20). Metrizamide and Percoll solutions were prepared in 0.25 M sucrose and densities were determined as described above. Gradients were prepared in 9/16 × 3.5-inch polyallomer centrifuge tubes just prior to use and placed on ice. A 2-ml cushion of 35% metrizamide was overlayed with 2 ml of 17% metrizamide and 4 ml of 6% Percoll. The tubes were filled to within 1 mm of the top with PNS (4.5 ml). The tubes were centrifuged in a SW41 rotor (Beckman) at 50,500 × *g* (27,000 rpm) for 25 min (excluding acceleration and deceleration time) at 4 °C with acceleration and deceleration settings of 7. Gradient fractions (0.8 ml) were collected from the bottom of the tube.

Assay of FPGS—FPGS activity was measured as described in a companion study (1). Tetrahydrofolate (50 μM) or dihydropteroate (50 μM), and L-[^{14}C]glutamate (500 μM; 1.25 μC) were used as the substrates. 0.1-ml sample fractions were used if obtained by differential centrifugation and 0.2 ml if obtained by the Percoll gradient method. Triton X-100 (0.1%) was normally added for the assay of mitochondrial extracts.

Marker Enzymes and Protein—Cytochrome *c* oxidase (22) and glutamate dehydrogenase (23) were used as mitochondrial membrane and matrix markers. Specific activity was calculated as the decrease of absorbance at 340 nm/min/mg of protein. β-Glucuronidase (lysosomal marker) was measured by a fluorescence method (24). Lactate dehydrogenase (25) was used as a cytosolic marker. Protein content was measured using the Bio-Rad protein assay using bovine serum albumin as a standard. A correction was made for the effects of Percoll on the protein assay by adding fractions obtained by gradient centrifugation in the absence of cell homogenate to protein standards.

Folate Accumulation and Distribution in CHO Cells—Cells were cultured as described previously (1, 2) in dialyzed minimal Eagle's medium/dFBS+GHT containing [^{3}H]folic acid or [^{3}H]folinic acid. After 24 h, cells were detached and subcellular fractionation was carried out as described previously. Polyglutamate chain lengths of cellular and subcellular folates were determined on duplicate samples as described previously (1).

RESULTS

Subcellular Distribution of FPGS Activity—After differential centrifugation, about 50% of WTT2 FPGS activity was associated with the crude mitochondrial fraction (Table I). The enzyme in this fraction was latent and its specific activity was over 4-fold higher than enzyme in the cytosolic fraction. Although the latency of FPGS was not as great as that of glutamate dehydrogenase, a mitochondrial matrix marker, the FPGS assay was carried out for 2 h without any attempt to preserve isotonic conditions, while the dehydrogenase was assayed over 10 min. The crude mitochondrial fraction from AUX-*human*-79 cells also contained a latent FPGS activity of higher specific activity than the cytosolic enzyme. Less than 5% of cellular FPGS in AUX-*coli* cells was associated with the mitochondrial fraction and this activity was not latent suggesting that it represented cytosolic contamination.

The crude nuclear fraction of WTT2 and AUX-*human*-79 cells contained some FPGS activity (data not shown). This fraction would contain any remaining whole cells and also entrapped organelles because of mild shearing of the ER during the nitrogen cavitation procedure (20, 24). No whole cells were observed under the microscope and this fraction also contained glutamate dehydrogenase and cytochrome c oxidase activities. Only trace levels of FPGS activity were detected in the nuclear fraction from AUX-*coli* cells.

TABLE I

Subcellular distribution of folylpolyglutamate synthetase activity in CHO cells

CHO cells were cultured in +GHT medium and subcellular fractions were prepared by nitrogen bomb cavitation and differential centrifugation as described under "Experimental Procedures." FPGS and glutamate dehydrogenase (GDH) activity in mitochondrial (Mito) and cytoplasmic (Cyto) fractions were measured as described under "Experimental Procedures." Values are the means of duplicate assays.

Cell	Fraction	FPGS activity[a]		GDH activity	
		pmol/h/mg	%	*ΔA/min/mg*	%
WTT2	Mito[b]	920 (356)	48	1.83 (0.18)	76
	Cyto	155	52	0.13	24
AUX-*human*-79	Mito	363 (152)	28	2.55 (0.19)	79
	Cyto	240	72	0.14	21
AUX-*coli*	Mito	95 (101)	5	2.58 (0.18)	89
	Cyto	547	95	0.15	11

[a] Tetrahydrofolate substrate for WTT2 and AUX-*human*-79, dihydropteroate for AUX-*coli*.

[b] Values in parentheses are assays of mitochondrial fractions in the absence of Triton X-100.

TABLE II

Subcellular distribution of folate in CHO cells

Folate-depleted cells were labeled in +GHT medium containing 8 nM [^{3}H]folinic acid for 40 h, and crude subcellular fractions were prepared by differential centrifugation as described under "Experimental Procedures."

Cell	Folate accumulation	Subcellular folate		
		Nuclear	Mitochondrial	Cytosolic
	pmol/10^6 cells		%	
WTT2	4.31	30	32	38
AUX-*human*-79	3.04	21	21	58
AUX-*coli*	4.01	2	3	95
AUXB1	0.018	18	29	53

Subcellular Distribution of Folate—After differential centrifugation, intracellular folate in WTT2 cells was approximately equally distributed between the crude nuclear, mitochondrial, and cytosolic fractions (Table II). About 50% of the folate in the postnuclear supernatant of WTT2 and about 25% of the folate in AUX-*human* cells were associated with the mitochondrial fraction. Only trace amounts of AUX-*coli* folate was associated with organelle fractions (Table II), ranging from 2 to 5% in three separate experiments. Although folate was associated with organelle fractions from AUXB1 cells, these cells contained only trace levels of labeled folate (Table II).

The distributions of folate and FPGS activity shown in Tables I and II have proven to be quite reproducible in multiple subcellular fractionation experiments, although occasionally (approximately 10% of experiments) the proportion of folate or FPGS in the crude mitochondrial fraction from WTT2 and AUX-*human* transfecants was reduced and the proportion in the nuclear fraction was increased. This phenomenon appeared to be due to an increased entrapment of organelles in the nuclear fraction, rather than incomplete cell breakage during the mild homogenization conditions, as nuclear fraction enrichment of folate and FPGS activity was never observed with AUX-*coli* cells.

The folylpolyglutamate chain length distributions of folate in the various fractions are shown in Table III. After a 40-h labeling period, folates in the crude mitochondrial fractions

TABLE III

Subcellular distribution of folylpolyglutamates in CHO cells

Folate-depleted cells were labeled in +GHT medium containing 8 nM [^{3}H]folinic acid for 40 h, and subcellular fractions were prepared by differential centrifugation as described in the legend to Table I. Folylpolyglutamates in mitochondrial (Mito) and cytoplasmic (Cyto) fractions and in the whole cell (Total) were identified as described under "Experimental Procedures."

Cell	Fraction	Polyglutamate chain length distribution									
		1	2	3	4	5	6	7	8	9	10
						%					
WTT2	Mito	1	1	2	2	4	15	47	25	3	0
	Cyto	2	2	3	5	22	35	23	8	1	0
	Total	2	1	2	2	10	24	41	18	1	0
AUX-*human*-79	Mito	1	1	2	1	3	9	29	40	12	2
	Cyto	2	1	2	3	12	33	25	17	4	1
	Total	1	1	1	2	6	19	28	32	9	1
AUX-*coli*	Mito	5	6	82	7	0	0	0	0	0	0
	Cyto	1	3	87	9	0	0	0	0	0	0
	Total	1	4	85	10	0	0	0	0	0	0
AUXB1	Mito	100	0	0	0	0	0	0	0	0	0
	Cyto	100	0	0	0	0	0	0	0	0	0
	Total	100	0	0	0	0	0	0	0	0	0

of WTT2 and AUX-*human* cells were of longer chain length than cytosolic folates. The distribution of whole cell folylpolyglutamates was intermediate between the mitochondrial and cytosolic patterns, and was consistent with that expected from the amount of labeled folate in each fraction (Table II), suggesting that hydrolysis of folylpolyglutamates did not occur to any significant extent during the fractionation procedure. The trace level of folate associated with the mitochondrial fraction of AUX-*coli* cells showed a similar chain length distribution to cytosolic folates but this fraction did contain a higher proportion of mono- and diglutamate (Table III). Folate in the nuclear fraction of cells closely resembled mitochondrial folate (data not shown), again suggesting that the crude nuclear fraction contained trapped organelles and that the proportion of folate associated with the mitochondria may be underestimated in these studies.

Density Gradient Fractionation of Folate and FPGS Activity—Postnuclear supernatants from CHO cells were further fractionated on a self-forming continuous Percoll density gradient (Fig. 1). Labeled folate from WTT2 and from AUX-*human*-79 cells was associated with two organelle peaks and with the cytosolic fraction while no organelle folate peaks were observed with AUX-*coli* extracts (Fig. 1*A*). The distribution of folate between the two organelle peaks was variable and most of the organelle folate was associated with the second, lighter peak. The mitochondrial marker enzymes cytochrome *c* oxidase and glutamate dehydrogenase co-eluted with the two organelle fractions, and some dehydrogenase activity was also present at the top of the gradient in the cytosolic fraction (Fig. 1*B*). β-Glucuronidase, a lysosomal marker, eluted one to two fractions after the second folate peak and in the cytosolic fraction. A significant proportion of the cell's lactate dehydrogenase, which was used as a cytosolic marker, was also associated with an organelle fraction, but this activity eluted later than β-glucuronidase (Fig. 1*B*). The subcellular distribution of protein in the different cell lines was similar.

FPGS activity showed a similar distribution to folate (Fig. 1*B*). In WTT2 and AUX-*human*-79 cells, FPGS was present in the heavy and light mitochondrial fractions and in the cytosolic fraction while no FPGS activity was detected in organelle fractions in AUX-*coli* cells. The specific activity of FPGS in the heavy mitochondrial fraction from WTT2 and

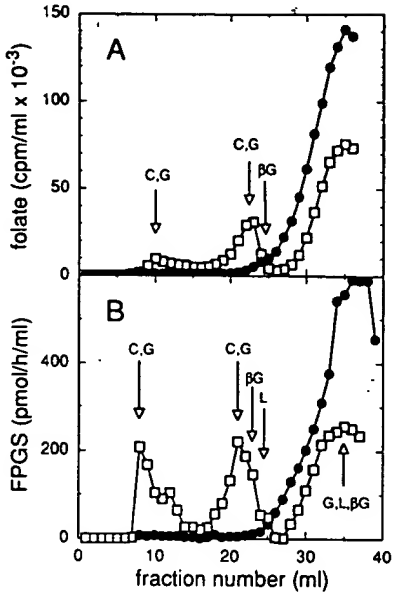

FIG. 1. **Percoll density gradient distribution of folate (*A*) and FPGS activity (*B*) in postnuclear supernatant of CHO cells.** AUX-*human*-79 (□——□) and AUX-*coli* cells (●——●) were incubated in MEM/dFBS+GHT medium containing [^{3}H]folinic acid (7 nM) for 24 h. Subcellular fractionation, Percoll density gradient centrifugation, and enzyme assays were carried out as described under "Experimental Procedures." Marker enzymes assayed were cytochrome *c* oxidase (*C*), glutamate dehydrogenase (*G*), β-glucuronidase (*βG*), and lactate dehydrogenase (*L*).

AUX-*human*-79 cells (about 2 nmol/h/mg of protein) was about 3-fold higher than in the light mitochondrial fraction (about 700 pmol/h/mg of protein).

FPGS and folate distributions were assessed in separate PNS samples as the presence of labeled folate complicates FPGS activity measurements. The relative elution positions of the different organelle markers shown in Fig. 1 were reproducible although the actual elution positions of individual organelle markers varied over a range of one to two fractions from experiment to experiment due to slight differences in the starting Percoll density.

As the self-forming Percoll density gradient did not completely resolve mitochondria and lysosomes, the PNS from WTT2 cells was also fractionated on a discontinuous Percoll-metrizamide gradient (20). A single mitochondrial fraction (interface of 17 and 35% metrizamide, Fig. 2, *fractions 3* and *4*) was well resolved from the lysosomal fraction (6% Percoll, 17% metrizamide interface, Fig. 2, *fractions 6–8*). Mitochondrial contamination of the lysosomal fraction was about 8%, as judged by the mitochondrial marker enzyme, glutamate dehydrogenase. About 90% of the organelle FPGS activity and folate were localized to the mitochondrial fraction, and less than 10% was associated with the lysosomal fraction. The latter is consistent with the extent of mitochondrial contamination of the lysosomal fraction although the possibility that lysosomes contain low levels of folate or FPGS activity can not be totally excluded. However, these data clearly demonstrate that most, if not all, cellular folate and FPGS is located in the mitochondria and the cytosol.

When aliquots of the same PNS from WTT2 cells were fractionated using both density gradient methods, the proportion of PNS folate associated with organelle fractions was 36% using the Percoll-metrizamide procedure while 38% was associated with the heavy and light mitochondrial fractions from the continuous Percoll gradient.

Mitochondrial Folate and FPGS Activity in AUX-human

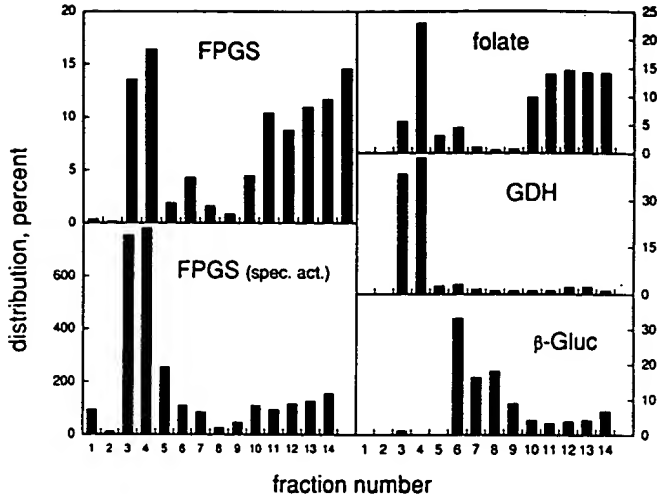

FIG. 2. **Percoll-metrizamide discontinuous density gradient distribution of folate, FPGS activity, and marker enzymes in postnuclear supernatant of CHO cells.** WTT2 cells were incubated in MEM/dFBS+GHT medium containing [^{3}H]folinic acid (7 nM) for 24 h. Subcellular fractionation, gradient centrifugation, and enzyme assays were carried out as described under "Experimental Procedures." Marker enzymes shown are glutamate dehydrogenase (*GDH*) and β-glucuronidase (*β-Gluc*). Enzyme and folate distributions are shown as percentage of total activity in each fraction, except for FPGS (specific activity) which is picomoles/h/mg of protein.

TABLE IV

Subcellular folate distribution in CHO transfectants

Folate-depleted cells were labeled in +GHT medium containing 7 nM [^{3}H]folinic acid for 24 h. Subcellular fractions were prepared by Percoll density gradient centrifugation as described in the legend to Fig. 1 and under "Experimental Procedures."

Cell	Folate uptake[a]	Mitochondrial folate	Folate uptake[b]	
			Mitochondria	Cytosol
		%		
WTT2	100	38	100	100
AUX-*human*-2	29	45	34	26
AUX-*human*-7	76	41	82	72
AUX-*human*-21	118	44	132	106
XUA-*human*-79	113	31	92	126
AUX-*coli*	150	0	0	242

[a] Net folate accumulation relative to WTT2 cells.
[b] Net subcellular folate accumulation relative to WTT2 cells.

Transfectants—AUXB1 cells transfected with human genomic DNA and expressing various levels of human FPGS activity were able to grow in the absence of glycine (2). Each of these transfectants accumulated folate in the mitochondria (Table IV) and AUX-*human*-7 and -21 cells showed a similar subcellular distribution of FPGS activity to that of AUX-*human*-79 cells (Fig. 1*B*). The proportion of mitochondrial folate in transfectants expressing lower levels of human FPGS appeared to be slightly higher than in AUX-*human*-79 cells (Table IV). Table IV also compares the effect of FPGS activity on folate levels in the mitochondria and cytosol. Under these conditions, cellular folate accumulation is limited primarily by transport (2), and FPGS activity became limiting only in the cytosol and mitochondria at very low levels (AUX-*human*-2). Cytosolic levels of folate in AUX-*coli* were higher than suggested by total cell folate because of an inability to accumulate folate in the mitochondria.

Table V shows the distribution of folylpolyglutamates in subcellular fractions from these cells. Mitochondrial folates were of longer glutamate chain length than cytosolic folates, an effect most pronounced in WTT2, the cell line expressing

TABLE V

Subcellular distribution of folylpolyglutamates in CHO cells

Folate-depleted cells were labeled in +GHT medium containing 7 nM [^{3}H]folinic acid for 24 h. Subcellular fractions were prepared by Percoll density gradient centrifugation as described in the legends to Fig. 1 and Table IV. Folylpolyglutamates in the cytoplasmic (Cyto) and heavy (Mito-1) and light (Mito-2) mitochondrial fractions were identified as described under "Experimental Procedures."

Cell	Fraction	Polyglutamate chain length distribution									
		1	2	3	4	5	6	7	8	9	10
						%					
WTT2	Mito-1	2	1	1	1	7	22	48	13	4	1
	Mito-2	1	0	1	2	12	29	42	10	2	1
	Cyto	3	1	1	3	24	42	20	6	1	0
AUX-*human*-2	Mito-2	4	7	16	26	42	4	1	0	0	0
	Cyto	9	11	18	29	27	4	1	0	0	0
AUX-*human*-7	Mito-1	1	1	4	5	49	36	3	1	0	0
	Mito-2	2	2	8	9	41	28	6	2	0	0
	Cyto	1	2	6	8	56	23	3	1	0	0
AUX-*human*-21	Mito-2	1	1	3	6	22	46	12	6	2	1
	Cyto	2	1	3	5	34	43	8	3	1	0
AUX-*human*-79	Mito-2	1	1	2	6	15	33	22	14	5	1
	Cyto	1	1	1	3	18	45	20	8	2	1

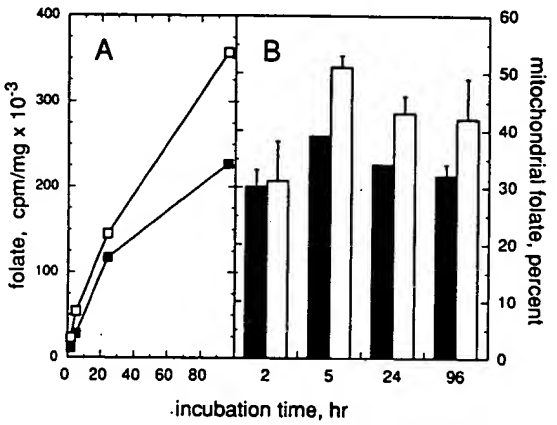

FIG. 3. **Effect of time on cellular folate (*A*) and mitochondrial folate (*B*) accumulation by WTT2 cells.** WTT2 cells were incubated in MEM/dFBS+GHT medium containing 7 nM [^{3}H]folinic acid (□——□) or 200 nM [^{3}H]folic acid (■——■) for the indicated times. Mitochondrial folate was measured after Percoll density gradient centrifugation as described under "Experimental Procedures." *Error bars* are standard deviations (n = 3).

the highest level of mitochondrial FPGS activity, and, as noted for whole cells (1, 2), the chain length was influenced by the level of FPGS activity. Chain lengths of folate in the heavy mitochondrial fraction were also slightly longer than in the light mitochondrial fraction, consistent with the higher specific activity of FPGS in this fraction. The folylpolyglutamate chain length distributions obtained after incubating cells with labeled folinate for 24 h (Table V) were slightly shorter than after a 40 h incubation (Table III).

Time Course of Mitochondrial Folate Accumulation—Growing WTT2 cells steadily accumulated folate and folinate over a 24 h labeling period but the rate of folate accumulation decreased slightly over the next 3 days (Fig. 3*A*). By 2 h, mitochondrial folate accounted for 30% of total cell folate and this proportion remained the same or increased slightly over the remainder of the 4-day incubation period (Fig. 3*B*). Mitochondrial folate accumulation was slightly higher in cells labeled with folinate than with folic acid.

DISCUSSION

Although a significant proportion of cellular folate is associated with the mitochondria (6, 8–12), there is little information concerning the nature of mitochondrial folates or the role of mitochondrial folate metabolism in one-carbon metabolism. Genetic studies have suggested that glycine is synthesized in the mitochondria (5) and it has been suggested that mitochondrial folate metabolism may also provide one-carbon moieties for cytosolic purine synthesis (14, 16, 26, 27).

In the current study, 30–50% of the folate in wild-type CHO cells and AUX-*human* transfectants, CHO cells expressing human FPGS activity, was found in the mitochondria and mitochondrial folates were of longer glutamate chain length than cytosolic folates, suggesting that the two folate pools are quite separate. A similar subcellular distribution was also observed for FPGS activity and the specific activity of the mitochondrial enzyme was higher than the cytosolic enzyme, which may explain the longer folylpolyglutamate derivatives in this organelle. Although FPGS was believed to be primarily a cytosolic enzyme, the possibility of a mitochondrial isozyme has been the subject of past speculation (3, 6, 28). The accumulation of mitochondrial folate was dependent on mitochondrial FPGS activity. AUX-*coli* transfectants lacked mitochondrial FPGS activity and mitochondrial folates, although mitochondria from these cells may have contained trace amounts of pteroylmono- and diglutamate.

The Percoll density gradient method used in this study separated mitochondria from cytosol and two mitochondrial peaks were detected (r = 1.11 and 1.07), implying a heterogeneity in CHO cell mitochondria, which has been observed previously (21). The lysosomal fraction was not well separated from the light mitochondrial fraction. However, the mitochondrial location of folate and FPGS activity was confirmed using a discontinuous hybrid Percoll-Metrizamide density gradient. Some lactate dehydrogenase activity was associated with an organelle fraction, as has been observed in other studies with mammalian cells (21, 29). Although the higher FPGS-specific activity in the heavy mitochondrial fraction may partly be due to some contamination of the light mitochondrial fraction with protein from other organelles, the slightly longer glutamate chain length of folate in the heavy mitochondrial fraction suggests that this fraction is more highly enriched in FPGS activity.

The levels of FPGS activity had little effect on the distribution of folate between mitochondria and cytosol. The time course of mitochondrial folate uptake by WTT2 cells indicated that folates were accumulated at a steady rate and there was no noticeable lag between total cell and mitochondrial folate uptake. This is consistent with entering pteroylmonoglutamates being transported into mitochondria before they are polyglutamylated by cytosolic FPGS. AUX-*human* transfectants with lower levels of FPGS activity tended to have a lower proportion of cytosolic folate, which may indicate a competition between folate transport into mitochondria and cytosolic FPGS activity. Overexpression of FPGS in the cytosol decreases the proportion of folate in the mitochondria.[2] The folylpolyglutamate distribution in the cell did vary with FPGS activity. Cells expressing higher enzyme levels metabolized folates to longer chain length derivatives in both the mitochondrial and cytosolic compartments.

The pteroyltriglutamates that accumulated in AUX-*coli* cells appeared to be as effective as longer derivatives in the synthesis of purine and thymidine, but not glycine (2). AUX-*coli* cells express high levels of *E. coli* FPGS activity in the cytosol but lack mitochondrial FPGS activity. The lack of mitochondrial FPGS was correlated with an inability to accumulate folate in mitochondria. Defective glycine synthesis was due to a lack of mitochondrial folate despite the presence of high levels of cytosolic folate. This further suggests that mitochondrial folate accumulation and metabolism is dependent on mitochondrial FPGS activity and that the mitochondrial and cytosolic folate pools are not in equilibrium. The non-equilibrium of folate one-carbon forms in mitochondria and cytosol has recently been demonstrated (30). The current study has also demonstrated that folylpolyglutamates are not co-transported with proteins into mitochondria.

Wild-type CHO cells, which can grow without glycine, purine, and thymidine, have both cytosolic and mitochondrial FPGS activity. The mutant AUXB1, which is auxotrophic for glycine, adenosine, and thymidine, was confirmed to be defective in both mitochondrial and cytosolic FPGS activity. The reversion frequency of AUXB1 is consistent with a single mutation, and revertants possess the wild-type phenotype (18, 31–33). All AUX-*human* transfectants, which were derived by transfection of AUXB1 with sheared human genomic DNA, contained mitochondrial folates and mitochondrial FPGS activity, suggesting that a single gene or closely linked genes code for the cytosolic and mitochondrial FPGS isozymes.

In a previous study comparing growth of cells in the absence of thymidine (2), we found that slightly lower levels of cellular pteroyltriglutamate supported half maximal growth rates of AUX-*coli* cells compared to folate levels in WTT2 and AUX-*human* cells. However, all the cellular folate in AUX-*coli* was in the cytosol while 30–50% of the cellular folate in the other cell lines tested was in the mitochondria. The cytosolic folate level that supported half-maximal growth in the absence of thymidine was essentially the same in AUX-*coli* and the other cell lines.

It has been suggested that oxidized pteroylmonoglutamates are the mitochondrial transport forms of folate and that reduced folates are not transported (34). However, data in a companion study (35) suggest that reduced folates are transported into the mitochondria. The unlikely possibility that folate transport into mitochondria requires a long chain folate derivative such as a tetra- or pentaglutamate cannot be completely excluded by these data. However, we have recently cloned a human FPGS cDNA (36), and expression of human FPGS in the cytosol of AUXB1 cells restores the cell's ability to grow in the absence of thymidine and purines, but the cell remains auxotrophic for glycine,[3] suggesting that longer chain length folylpolyglutamates do not enter the mitochondria. We have also recently shown that direction of the *E. coli* FPGS to the mitochondria of AUXB1 cells restores cell growth in the absence of glycine.[2]

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